FEMS Microbiology Letters, 362, 2015, fnv049

doi: 10.1093/femsle/fnv049
Advance Access Publication Date: 2 April 2015
Research Letter

RESEARCH LETTER – Pathogens & Pathogenicity

Allicin from garlic inhibits the biofilm formation and urease activity of *Proteus mirabilis in vitro*

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One sentence summary: Allicin from garlic inhibits the growth, biofilm formation and urease activity of *Proteus mirabilis in vitro*.

Editor: David Clarke

ABSTRACT

Several virulence factors contribute to the pathogenesis of *Proteus mirabilis*. This study determined the inhibitory effects of allicin on urease, hemolysin and biofilm of *P. mirabilis* ATCC 12453 and its antimicrobial activity against 20 clinical isolates of *P. mirabilis*. Allicin did not inhibit hemolysin, whereas it did inhibit relative urease activity in both pre-lysed (half-maximum inhibitory concentration, IC50 = 4.15 μg) and intact cells (IC50 = 21 μg) in a concentration-dependent manner. Allicin at sub-minimum inhibitory concentrations (2–32 μg mL^{-1}) showed no significant effects on the growth of the bacteria ($P > 0.05$), but it reduced biofilm development in a concentration-dependent manner ($P < 0.001$). A higher concentration of allicin was needed to inhibit the established biofilms. Using the microdilution technique, the MIC90 and MBC90 values of allicin against *P. mirabilis* isolates were determined to be 128 and 512 μg mL^{-1}, respectively. The results suggest that allicin could have clinical applications in controlling *P. mirabilis* infections.

Keywords: allicin; *Proteus mirabilis*; antimicrobial; urease; hemolysin; biofilm

INTRODUCTION

Proteus mirabilis, a member of the Enterobacteriaceae family, is one of the leading causes of urinary tract infections (UTIs) in patients with indwelling catheters or structural abnormalities in the urinary tract (Coker *et al.* 2000). *Proteus mirabilis* UTIs are most commonly associated with urinary tract obstruction, blockage of urinary catheters, bladder and kidney stone formation, and bacteriuria. Intrinsically, *P. mirabilis* UTIs are often persistent and difficult to treat (Rozalski, Sidorczyk and Kotelko 1997). Furthermore, the development of extended spectrum beta-lactamase producing and multiple drug resistant strains of *P.* mirabilis (Mokracka, Gruszczynska and Kaznowski 2012; Kurihara *et al.* 2013) have made treating *P. mirabilis* infections more difficult. Therefore, finding antibiotics with new modes of action and alternatives to commonly used antimicrobial therapies for

Received: 24 November 2014; **Accepted:** 25 March 2015

treating *P. mirabilis* infections is urgently needed. Neutralizing or suppressing the expression of the pathogen's virulence factors is desired to overcome infections (Fernebro 2011); it attenuates the pathogenicity of the bacterium and makes it easier for the host innate immune system to clear the infection (Fernebro 2011). *Proteus mirabilis* produces several potential virulence factors responsible for the pathogenicity of the organism, including urease and hemolysin (Rozalski, Sidorczyk and Kotelko 1997; Coker *et al.* 2000). Urease is produced by virtually all clinical strains of the bacterium (Rozalski, Sidorczyk and Kotelko 1997; Stankowska, Kwinkowski and Kaca 2008) and catalyzes the hydrolysis of urea to ammonia and carbon dioxide. Ammonia, a strongly alkaline agent, is a major cause of damage to host tissues. It has been shown that ammonia derivatives, such as ammonium hydroxide and monochloramine, are directly toxic to mammalian cells (Burne and Chen 2000). On the other hand, accumulation of ammonium increases the pH in surrounding tissues, thereby affecting the enzyme activities of the body system and triggering stone formation in the bladder and kidney (Griffith, Musher and Itin 1976; Burne and Chen 2000). Similarly, it has been demonstrated using animal models that urease-deficient *P. mirabilis* strains are less virulent and have lower 50% lethal dose (LD50) in comparison to parent strains (Jones *et al.* 1990). These findings are further supported by the fact that treating the animals with acetohydroxamic acid, a potent urease inhibitor, reduces the severity of *P. mirabilis* infection (Musher *et al.* 1975).

Hemolysin, another host damaging virulence factor of *P. mirabilis*, has cytotoxic effects on urinary tract epithelial cells (Mobley *et al.* 1991). It has been shown that hemolytic activity correlates with the cell invasiveness of *P. mirabilis* strains *in vitro* (Rozalski, Dlugonska and Kotelko 1986). This was further confirmed using a mouse model in which it was shown that the hemolysin-negative strains had higher LD50 than hemolysin-positive strains (Swihart and Welch 1990).

Biofilms, in particular crystalline biofilms, play a significant role in *P. mirabilis* UTIs. Biofilms protect organisms from the host immune system and antimicrobials (Jacobsen and Shirtliff 2011). Crystalline biofilms make *P. mirabilis* UTIs more complicated. The crystallization of biofilms using magnesium phosphate and calcium phosphate leads to biofilm encrustation and blockage of indwelling catheters in patients with UTI (Mobley and Warren 1987).

According to the data presented above, urease, hemolysin and biofilm of *P. mirabilis* can be excellent therapeutic targets for treatment of *P. mirabilis* UTIs. This study aimed mainly to investigate the *in vitro* efficacy of allicin in neutralizing urease and hemolysin and to evaluate the effects of sub-inhibitory concentrations of allicin on biofilm formation in *P. mirabilis*. The antimicrobial activity of allicin against *P. mirabilis* strains was further evaluated. Allicin as the active principle of garlic extract possesses a number of pharmacological effects including antimicrobial activities (Harris *et al.* 2001). It has been shown that allicin could interfere with bacterial virulence factors in sub-minimum inhibitory concentrations (sub-MIC; Arzanlou and Bohlooli 2010a; Arzanlou *et al.* 2011; Leng *et al.* 2011).

MATERIALS AND METHODS

Bacterial strains, chemicals and media

A total of 20 clinical strains and one standard strain of *P. mirabilis* (ATCC 12453) were used in this study. Clinical strains were isolated from patients with UTIs admitted to a major university-affiliated teaching hospital (Imam Hospital, Ardabil University of Medical Sciences) and further confirmed using standard biochemical tests. Bacteria were cultured on trypticase soy agar (TSA) slants for daily use and stored in a trypticase soy broth medium along with 15% glycerol, at –80°C for subsequent uses. All chemicals and media were purchased from Merck (Darmstadt, Germany), except where noted.

Allicin preparation and quantification

Allicin (purity ≥95%) was purified from garlic extract using the semi-preparative HPLC method and quantified by analytical HPLC as described in detail by authors elsewhere (Arzanlou and Bohlooli 2010b; Arzanlou, Bohlooli and Ranjbar-Omid 2015).

Antimicrobial susceptibility assay

The antimicrobial activity assay of allicin against *P. mirabilis* was performed using a microdilution method according to procedures recommended by the Clinical Laboratory Standards Institute (CLSI 2011). Briefly, 2-fold serial dilutions of allicin were prepared in sterile Mueller Hinton Broth (MHB) for a testing concentration range of 2–1024 μg mL^{-1}. Then 100 μL from each dilution was transferred into the well of a microtiter plate and inoculated with 5 μL of standardized (1.5×10^7 CFU mL^{-1}) cell suspension. Plates were incubated at 37°C overnight, and the lowest concentration of allicin that prevented visible growth was recorded as the MIC. Minimum bactericidal concentration (MBC) was determined by sub-culturing 10 μL of broth from wells with no visible growth on TSA plates. The lowest concentration of allicin that killed 99.9% of the original inoculum was considered as MBC.

Urease production and activity assay

A total of 50 μL of overnight culture of *P. mirabilis* ATCC 12453 in MHB were transferred into 10 mL sterile MHB and additionally incubated 18 h at 37°C with constant shaking. The cells were pelleted by centrifugation at 1258 *g* for 15 min (4°C). The pellet was washed three times with 10 mM K_2HPO_4 solution and re-suspended in 2 mL of the same solution. Thereafter, to release the urease, bacteria were sonicated for 90 s with 0.5 cycles at 100% amplitude using an ultrasonicator (UP200H, Hielscher Ultrasonics, Teltow, Germany) in an ice container. The resulting bacterial lysate was used for urease activity assay. Urease activity assay was performed in a microtiter plate using the phenol red colorimetric method in a mixture containing 10 mM K_2HPO_4 solution (pH 6.2), 0.002% phenol red and 500 mM urea (assay reagent). The increase in absorbance at 570 nm was recorded using a microplate reader (BioTek, USA) (Goldie *et al.* 1989; Tanaka, Kawase and Tani 2003). Activity was calculated using a standard curve relating to NH_3 solutions of known concentration (Norris and Brocklehurst 1976) and expressed as mmol NH_3 min^{-1} mg^{-1} protein (Bauerfeind *et al.* 1997). In all experiments, the freshly prepared bacterial lysate was used. To obtain solutions with the same urease activity, the lysate was diluted to a defined specific activity (5.66 mmol NH3 min^{-1} mg^{-1} protein) with 10 mM K_2HPO_4 and then the same volume used in different experiments.

Inhibition of urease activity

To evaluate the inhibitory effects of allicin on urease, 15 μL of bacterial lysates (approximately 1.7 mg protein) were pre-incubated with various concentrations of allicin (1, 3, 5 and 7 μg), for 30 min at 37°C, and then the urease activity was measured as described above. The positive control for the assay was

prepared in the same manner, but without the allicin. The absorbance values of each reaction were plotted against the time, and the residual activity was calculated by dividing the slope of the regression line obtained for each reaction by the slope of the positive control. Similar experiments were done to evaluate the inhibitory effects of iodoacetic acid (IAA) as a known urease inhibitor.

In parallel, to ensure that the color change was not due to allicin or urea, negative controls consisting of allicin without bacterial lysate or controls free of urea (with and without allicin) were included.

Inhibition of urease inside the bacteria

The bacterial cell suspension with an optical density of 0.7 at a wave length of 620 nm in 10 mM K_2HPO_4 solution (pH 6.2) was incubated with 10, 25, 50 μg concentrations of allicin for 1 h at room temperature. Thereafter, the bacteria were harvested by centrifugation at 1258 *g* for 10 min and washed three times with the same buffer to remove probable remaining allicin. Then, the bacteria were resuspended in 10 mM K_2HPO_4 solution (pH 6.2) and lysed with an ultrasonicator, and the urease activity was assayed as described above.

Effects of a reducing agent on urease re-activation

An experiment was conducted to elucidate whether the reducing agent dithiothreitol (DTT) could restore urease activity after incubation with allicin. A total of 15 μL of bacterial lysates (approximately 1.7 mg protein) was pre-incubated with different inhibitory agents for 30 min at 37°C, and then the mixture was further incubated in the presence of 4 μL DTT (5 mM final concentration) for 30 min at room temperature. The activity of urease was measured as described above, and residual activity was expressed as relative activity. In order to elucidate the molecular mechanism of urease inhibition, the same experiments were conducted on urease inhibited by IAA. Minimum concentrations of agents which fully inhibit urease were used in all experiments.

Hemolysin production and hemolytic activity assay

Culture and disruption of *P. mirabilis* ATCC 12453 were performed in the same manner as urease production, except that the culture was carried out without shaking and the lysate was prepared in normal saline. The bacterial lysate was used for hemolytic activity assay which was carried out using human red blood cells (RBCs) according to the method previously described by authors (Arzanlou and Bohlooli 2010a). Briefly, hemolytic activity was determined with serial dilutions of bacterial lysate in normal saline. Washed human type O RBCs were added to the tubes to yield a final concentration of 2%. All tubes were incubated at 37°C for 30 min. The remaining intact erythrocytes were removed by gentle centrifugation at 805 *g* for 2 min. The absorbance of released hemoglobin was determined at 541 nm using a spectrophotometer. The amount of hemolysin that produced 50% hemolysis was defined as 1 hemolytic unit (HU). Controls containing 2% erythrocytes and de-ionized water which were considered 100% hemolysis were used to determine the percentage of hemolysis. All experiments were conducted in triplicate.

Assay of hemolytic activity inhibition

Inhibition studies of hemolytic activity in the presence of allicin were carried out according to the method previously described by authors (Arzanlou and Bohlooli 2010a). Bacterial lysates (200 μL; ~1 HU) were pre-incubated with various concentrations (5, 10, 15, 20 μg) of allicin for 15 min at ambient temperature. Washed RBCs were added to yield a final concentration of 2%. All tubes were incubated at 37°C for 30 min. To remove the intact RBCs, tubes were centrifuged gently at 805 *g* for 2 min. The absorbance of the released hemoglobin was determined at 541 nm. The positive control for the assay was prepared in the same manner, but without allicin. Activity without the inhibitor was considered to be 100%, and the residual activity at each concentration of allicin was determined relative to this value. In parallel, to ensure that the lysis was not due to the test material, a negative control consisting of allicin without bacterial lysate was included.

Growth curve

A suspension of *P. mirabilis* ATCC 12453 and two representative clinical strains (with strong biofilm-forming abilities) with turbidities equal to McFarland turbidity standard No. 0.5 were prepared in normal saline. A total of 100 μL of the suspensions was transferred into 10 mL sterile MHB (1.5×10^7 CFU mL^{-1}) in 100 mL Erlenmeyer flasks containing 2, 4, 8, 16, 32, 64 and 128 μg mL^{-1} allicin. In parallel, a control without allicin was included. The flasks were incubated at 37 °C with constant shaking under aerobic conditions. The growth of cells was measured by reading the optical density (620 nm) of 100 μL samples at 2 h intervals, up to 18 h. Optical density values from triplicate experiments were averaged and plotted against time points.

Screening of strains for biofilm formation

To select the strains with strong biofilm-forming ability, all strains ($n = 21$) were tested for their ability to form biofilm using polystyrene flat-bottomed microtiter plates (Orange Scientific, Belgium) as described by O'Toole *et al.* 1999 with some modifications. Briefly, 200 μL of sterile MHB was dispensed into wells of a microtiter plate. The wells were inoculated with 10 μL of standardized (1.5×10^7 CFU mL^{-1}) cell suspension. The plates were incubated for 18 h at 37°C. Thereafter, the biofilm-coated wells of microtiter plates were washed three times with 300 μL of sterile PBS (pH 7.4) to remove non-adherent cells, dried in an inverted position in room temperature. Afterwards, each of the washed wells was stained with 200 μL of 0.2% safranin aqueous solution for 2 min and then washed three times with 300 μL of sterile distilled water to remove excess dye. The bacteria were decolorized with 200 μL of 95% ethanol for 15 min, then 150 μL of the resolubilized dyes were transferred to a new well, and the amount of the safranin stain was measured with the microtiter plate reader at a 492 nm wavelength. To minimize background interface, controls including bacteria-free medium were included in each experiment, and the absorbance values for the controls were subtracted from the values for the test wells and defined as biofilm forming ability. All strains were tested in quadruplicate in three independent experiments.

Biofilm-forming ability was defined as strong ($OD \geq 0.3$), moderate ($0.2 \leq OD < 0.3$), weak ($0.2 > OD \geq 0.1$) and negative ($OD < 0.1$) (Wasfi *et al.* 2012).

Effects on biofilm formation

In this study, *P. mirabilis* ATCC 12453 and two clinical strains with strong biofilm-forming abilities were included. The effect of sub-MIC concentrations of allicin on biofilm formation was evaluated according to the method described above, except that bacteria were cultured in the presence of defined concentrations (2, 4, 8, 16 and 32 μg mL^{-1}) of allicin. Biofilm formation without allicin was considered to be 100%, and the percentage of biofilm inhibition at each concentration of allicin was determined relative to this value.

Effects on established biofilm

The effect of allicin on established biofilms was investigated as described by others with some modifications (Nostro *et al.* 2007). Bacteria were grown as biofilm using the same method described above. Planktonic phase cells were removed and the wells were washed three times with PBS. Then, the wells were filled with 200 μL of 2-fold multiples of allicin in MHB, ranging from MIC to 5-fold multiples of MIC. The plates were incubated 24 h at 37°C. The biofilm inhibitory concentration (BIC) was defined as the lowest concentration where no visible growth was observed. Samples of biofilms from the bottom of these wells were scarified by a metal loop, spread over the surface of TSA plates, and incubated for 72 h at 37°C. The biofilm eradication concentration (BEC) was taken as the minimum concentration at which viable cells could no longer be recovered from the biofilm directly.

Statistical analysis

All experiments were repeated at least three times and obtained data was presented as mean ± SD. Statistical analysis was performed using Student's *t*-test, and $P < 0.05$ was considered significant. The half-maximum inhibitory concentration (IC50) values for the inhibition study of urease activity were calculated by fitting data to the Hill equation using SigmaPlot (version 11.0) software (Systat Software).

RESULTS

Purification of allicin

A typical representative chromatogram of purified allicin is shown in Fig. 1. As shown in the chromatogram, purified allicin

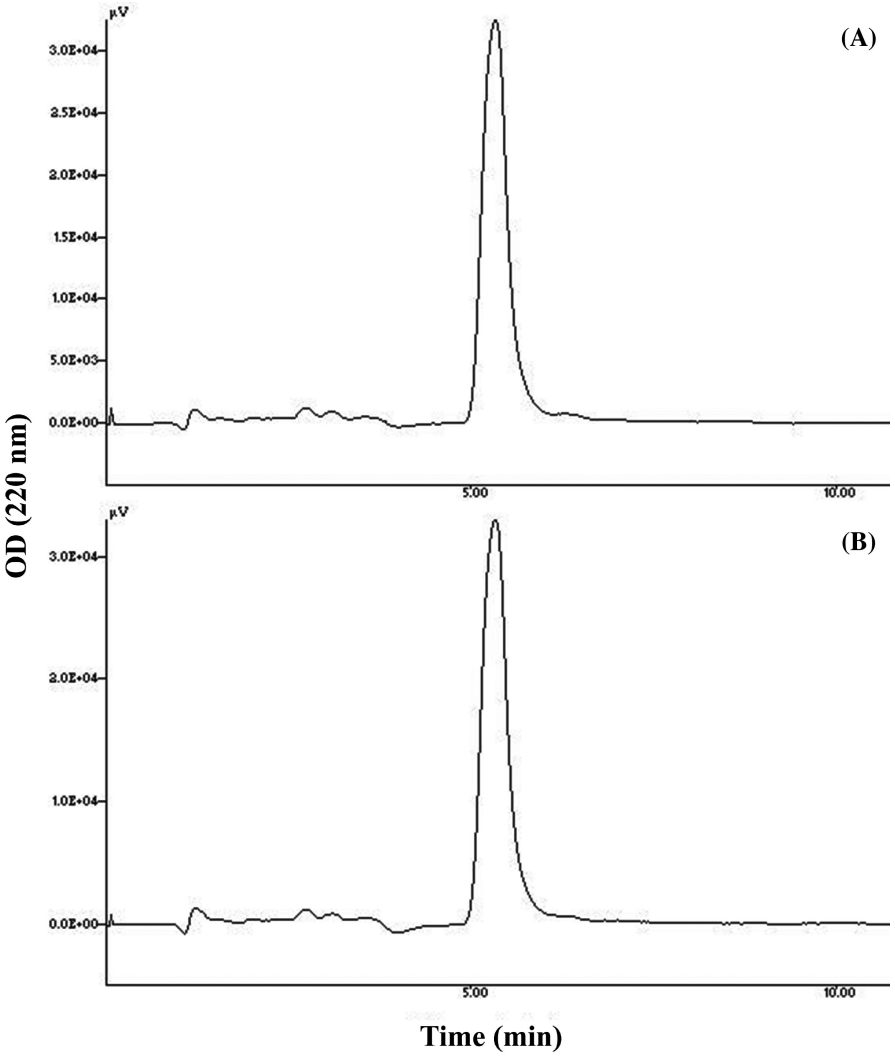

Figure 1. Representative chromatogram of purified (**A**) and standard (**B**) allicin using analytical HPLC.

Table 1. Susceptibility of *P. mirabilis* clinical strains to purified allicin.

MIC (μg mL^{-1})	Strains *n* (%)	MBC (μg mL^{-1})	Strains *n* (%)
64	3 (14.3)	64	–
128	18 (85.7)	128	1 (4.7)
256	–	256	9 (42.8)
512	–	512	11 (52.4)
Total	21 (100)	Total	21 (100)

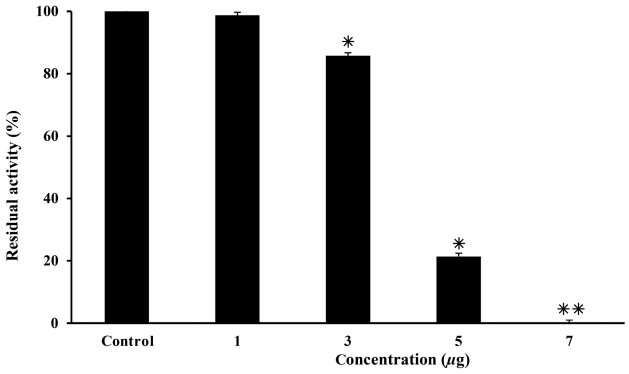

Figure 2. Inhibition of urease activity in bacterial cell lysate using allicin. Bacterial cell lysate was incubated for 30 min with defined concentrations of allicin and the urease activity was assayed. Results were obtained from three independent triplicate experiments. Relative activity was calculated in comparison to the allicin-free control and expressed as mean ± SD. *statistically significant ($P < 0.05$) compared with the allicin-free control. **statistically significant ($P < 0.001$) compared with the allicin-free control.

yielded a single peak with a retention time of 6 min, inconsistent with standard allicin, and constituted ≥ 95% of the total detectable peak areas.

Antimicrobial susceptibility assay

The results showed that allicin was active against *P. mirabilis* strains. The tested clinical isolates revealed the MICs of either 64 or 128 μg mL^{-1}and MBCs of either 128 or 512 μg mL^{-1} (Table 1). The MIC 90 and MBC 90 for tested strains were 128 and 512 μg mL^{-1}, respectively. The MIC and MBC for the control strain tested (*P. mirabilis* ATCC 12453) were 64 and 128 μg mL^{-1}, respectively.

Inhibition of urease activity in bacterial lysate

The results showed that allicin decreased urease relative activity in a concentration-dependent manner. Fig. 2 represents the decrease of urease activity after 30 min pre-incubation with increasing concentrations of allicin. IC50 was achieved with 4.15 μg of allicin and 7 μg completely inhibited urease compared with the allicin-free control. Urea-free controls showed no activity with or without allicin (data are not shown). Similar to allicin, IAA inhibited urease activity in a concentration-dependent manner, and maximum inhibition was achieved at 8 μg (Fig. 3).

Inhibition of urease activity inside the bacteria

The results showed that allicin easily diffused into bacteria and inhibited urease relative activity in a concentration-dependent manner. Fig. 4 shows that urease activity decreased as allicin concentrations increased after 30 min pre-incubation of intact

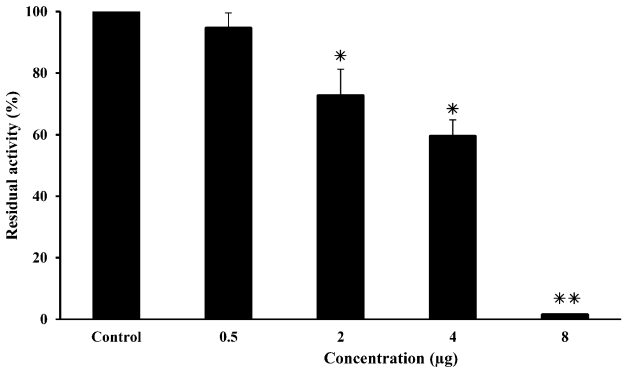

Figure 3. Inhibition of urease activity in bacterial cell lysate using IAA. Bacterial cell lysate was incubated for 30 min with defined concentrations of allicin and the urease activity was assayed. Results were obtained from three independent triplicate experiments. Relative activity was calculated in comparison to the IAA-free control and expressed as mean ± SD. *statistically significant ($P < 0.05$) compared with the IAA-free control. **statistically significant ($P < 0.001$) compared with the IAA-free control.

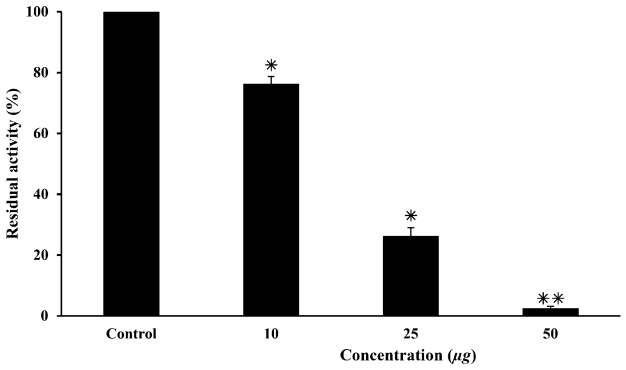

Figure 4. Inhibition of urease enzyme inside intact bacterial cell using allicin. The bacterial cells were disrupted after exposure to defined concentrations of allicin (30 min at room temperature) and the urease activity was assayed. Residual activity was calculated in comparison with the allicin-free control and expressed as mean ± SD. Results were obtained from three independent triplicate experiments. *statistically significant ($P < 0.05$) compared with the allicin-free control. **statistically significant ($P < 0.001$) compared with the allicin-free control.

cells with allicin. The IC50 was 21.01 μg, while 50 μg completely inhibited urease.

Effects of a reducing agent on urease inhibition by allicin

Results indicated that the incubation of allicin-treated urease (7 μg) with 5 mM DTT restored its enzymatic activity ($P > 0.001$). DTT did not restore the inhibitory effect of IAA (Fig. 5).

Inhibition of hemolytic activity

As demonstrated in Fig. 6, allicin did inhibit the hemolytic activity of *P. mirabilis* hemolysin ($P > 0.05$).

Screening of strains for biofilm formation

Tested strains showed variable results in the aspect of biofilm formation. Most clinical strains as well as the *P. mirabilis* strain ATCC 12453 were put into the strong biofilm-former category (75, 20 and 5% were strong, moderate and weak biofilm-formers,

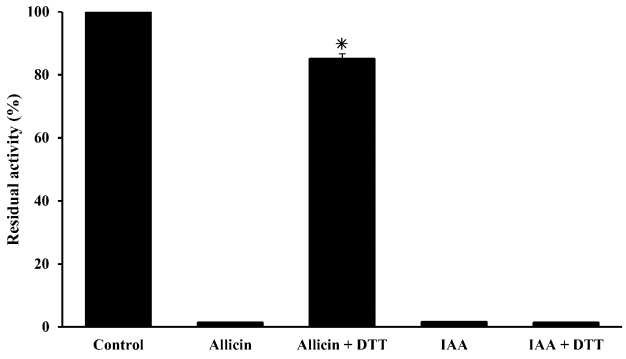

Figure 5. Effect of DTT on restoring urease activity after incubation with allicin and IAA. Bacterial cell lysate was incubated for 30 min with 7 and 8 μg concentrations of allicin and IAA, respectively. The reactions were further incubated with DTT (5 mM) for 30 min. Residual activity was calculated in comparison with DTT-free controls and expressed as mean ± SD. Results were obtained from three independent triplicate experiments. DTT did not restore the inhibitory effect of IAA ($P > 0.05$). *statistically significant ($P < 0.001$) compared with the DTT-free control.

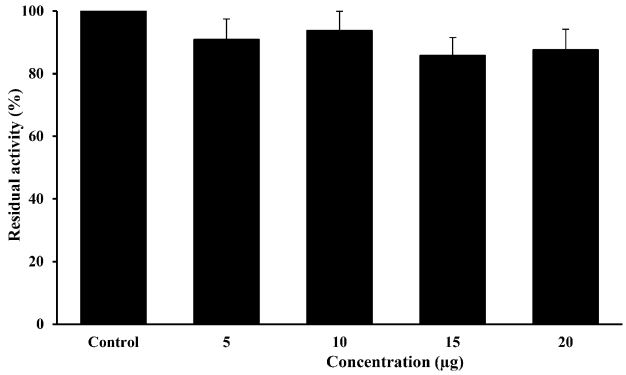

Figure 6. Inhibitory effects of allicin on the hemolytic activity of *P. mirabilis*. Bacterial lysate was pre-incubated for 30 min with various concentrations of pure allicin (5, 10, 15, 20 μg). Hemolytic activity was measured and residual activity was calculated in comparison with the allicin-free control. Results were obtained from three independent triplicate experiments and expressed as mean ± SD. Allicin did not inhibit the hemolysin ($P > 0.05$) compared with the allicin-free control.

respectively). Two isolates from the strongest biofilm-forming *P. mirabilis* strains were selected for biofilm inhibition studies.

Effects of sub-inhibitory concentrations of allicin on *P. mirabilis* growth

Fig. 7 shows the growth curve of *P. mirabilis* strain ATCC 12453 in the presence of increasing concentrations of allicin. As shown in the figure, sub-MIC concentrations of allicin at 2–32 μg mL^{-1} had no significant influence on *P. mirabilis* growth ($P > 0.05$) at 18 h after the inoculation of bacteria into the culture medium. Allicin at 32 μg mL^{-1} delayed bacteria growth in the first hours after the inoculation of bacteria into the culture medium; but increasing the incubation time up to 18 h produced no significant difference in growth compared with the allicin-free control ($P > 0.05$). At concentrations equal to or above the MIC value, allicin completely inhibited growth. The growth of two clinical strains tested showed similar responses to allicin (data are not shown).

Effects of allicin on biofilm development

Allicin reduced biofilm formation in a concentration-dependent manner in *P. mirabilis* (Fig. 8). Allicin at 16 and 32 μg mL^{-1} showed the maximum reduction in biofilm development by 28.9% ± 0.2 and 33.8% ± 1.0 in *P. mirabilis* strain ATCC 12453, 27.5% ± 0.3 and 35.1% ± 1.0 in clinical isolate 1, and 16.4% ± 0.1 and 25.7% ± 0.1 in clinical isolate 2.

Effects of allicin on established biofilm

The BIC and BEC of allicin for tested *P. mirabilis* strain ATCC 12453 were 2-fold (256 μg mL^{-1}) and 4-fold (512 μg mL^{-1}) greater than its MBC, respectively. The MIC and MBC values for this strain were 64 and 128 μg mL^{-1} in MHB, respectively.

DISCUSSION

The pathogenesis of *P. mirabilis* is multifactorial, and plenty of virulence factors work together to cause diseases. Inhibiting virulence factors could attenuate bacterium and enable the host immune system to combat disease. Wide varieties of natural products have been studied to inhibit *P. mirabilis* virulence factors (Wang *et al.* 2006; Carpinella *et al.* 2011; Cock and van Vuuren 2014; Packiavathy *et al.* 2014). The current study reports the inhibitory effects of allicin on the urease, hemolysin and biofilm of *P. mirabilis* as well as its antimicrobial activity against the bacterium. Allicin is a natural product derived from garlic (*Allium sativum*). It was found to inhibit the growth of a wide range of bacteria; it also showed antiparasitic, antifungal and antiviral activity *in vitro* (Harris *et al.* 2001). Allicin is commercially available, but its instability and high price limit the amount that can be used. Several previous studies on the antimicrobial activity of allicin have been carried out essentially with garlic extract rather than purified allicin. In this study, purified allicin was used in all experiments. The results showed that allicin inhibits the growth of the majority of *P. mirabilis* strains at concentrations lower than the MIC values previously reported to other Gram-negative bacilli, *Pseudomonas aeruginosa*, and oral anaerobic bacteria (Cai *et al.* 2007; Bachrach *et al.* 2011). Urease is suggested as an important target in the development of drugs for treating infection caused by urease positive bacteria (Follmer 2010). The activity of *P. mirabilis* ureases can be inhibited by substrate structural analogs of urea (such as hydroxyurea, thiourea, methylurea, and hydroxamic acid derivatives and phosphotriamides) and proton-pump inhibitors (such as rabeprazole, omeprazole and lansoprazole) (Follmer 2010). Proton-pump inhibitors indirectly inhibit urease. In a low pH environment, they are converted to sulfonamides which, in turn, inhibit urease via cysteine modification at the active site of the enzyme (Nagata *et al.* 1993; Tsuchiya *et al.* 1995). Unique features of bacterial urease are its association with nickel and its large number of cysteine residues (Rando *et al.* 1990). Cysteine-319 is key residue at the active site of *P. mirabilis* urease (Sriwanthana and Mobley 1993). Results of the current study showed that allicin efficiently inhibited *P. mirabilis* urease, likely via a mechanism involving the formation of a disulfide bond with reactive cysteine residue at the urease active site. This mechanism was further confirmed by the restoration of activity of the enzyme in the presence of a reducing agent, DTT. This is consistent with the proposed mode of action for allicin to form a disulfide bond with cysteine residue at the active site of enzymes, and consequently to inhibit the catalytic activity of urease (Rabinkov *et al.* 1998). Previous studies on jack bean urease (Juszkiewicz *et al.* 2003), microbial

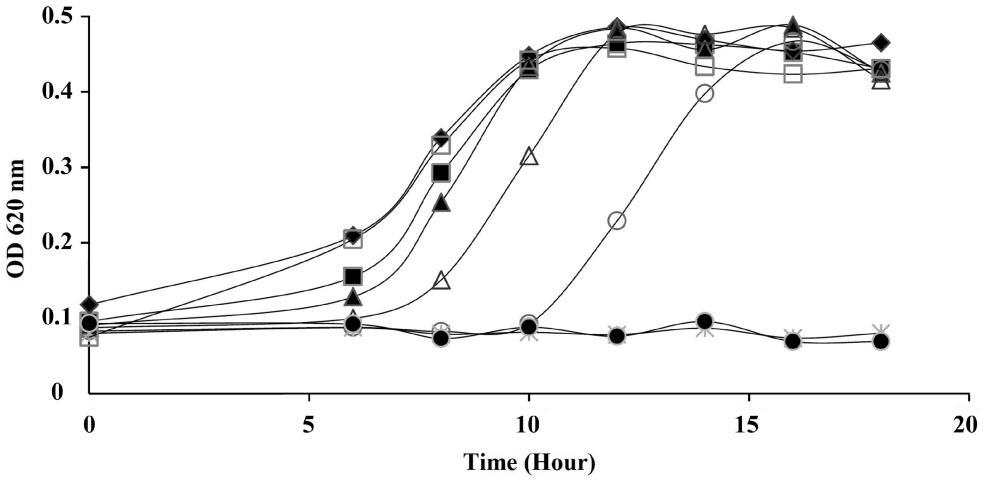

Figure 7. Growth curves of *P. mirabilis* strain ATCC 12453 cultured in MHB in presence of allicin. Allicin concentrations (μg mL^{-1}): (filled square) 2, (filled diamond) 4, (filled triangle) 8, (open triangle) 16, (open circle) 32, (star) 64, (filled circle) 128, (open square) Control without allicin. The OD 620 nm was not statistically significant ($P > 0.05$) compared with the allicin-free control at 18 h in sub-MIC concentrations.

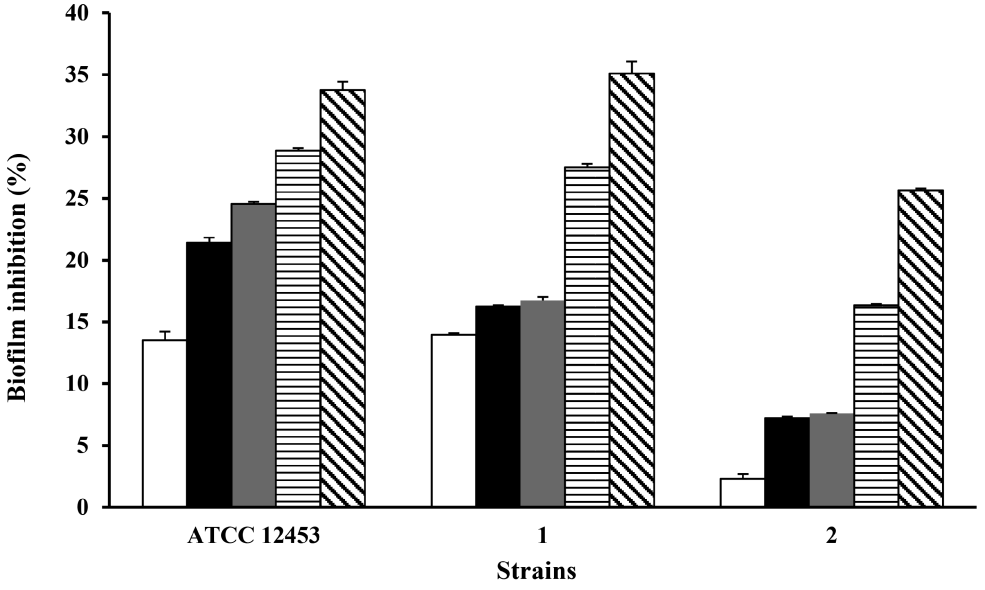

Figure 8. Inhibition of biofilm formation by sub-MIC concentrations of allicin. Bacteria were cultured in the presence of sub-MIC concentrations (μg mL^{-1}) of allicin [(black filled square) 2, (open square) 4, (gray filled square) 8, (square filled with diagonal lines) 16, (square filled with horizontal lines) 32] for 18 h and biofilm development was assayed. Inhibition percentage was calculated in comparison with the allicin-free control and expressed as mean ± SD. Results were obtained from three independent triplicate experiments. Statistically allicin (all concentrations) did inhibit biofilm formation compared with the allicin-free control ($P < 0.001$).

SH-containing enzymes (Wills 1956; Wallock-Richards *et al.* 2014) and toxins (Arzanlou and Bohlooli 2010a; Arzanlou *et al.* 2011) suggested the same mode of action for allicin. This mechanism of action was further confirmed by comparison with IAA as an irreversible inhibitor of cysteine containing enzymes. IAA inhibits urease activity with the mechanism of inhibition occurring from the alkylation of the catalytic cysteine residue (Upadhyay 2012). As expected, DTT did not restore its activity.

Inhibition of urease inside the intact bacteria revealed that allicin efficiently diffuses into cytoplasm and inhibits urease. Previous studies indicated that allicin could easily pass through biological membranes (Miron *et al.* 2000). Passing through bacterial envelopes could be an excellent advantage of allicin over other urease inhibitors. A few urease inhibitors such as hydroxamic acid derivatives are known to penetrate the *Helicobacter pylori* cells and inhibit urease activity inside the bacteria (Nakamura *et al.* 1998; Follmer 2010).

Significantly higher concentrations of allicin were required to completely inhibit urease activity inside intact cells compared to pre-lysed *P. mirabilis*. These results are consistent with previous reports showing that complete inhibition of *Entamoeba histolytica* cysteine proteases and *Streptococcus pneumonia* pneumolysin in intact organisms occurs at higher concentrations compared with pre-lysed organisms (Ankri *et al.* 1997; Arzanlou *et al.* 2011). Complete inhibition for both was achieved at concentrations lower than MIC values as determined for *P. mirabilis* in this study. The low inhibitory concentration of allicin toward *P. mirabilis* urease is likely due to some allicin

features such as a high affinity toward -SH groups (Rabinkov *et al.* 1998; Arzanlou and Bohlooli 2010a; Arzanlou *et al.* 2011), low molecular weight and simple structure which make it easier to enter the active sites of sulfhydryl enzymes/toxins. However, several other factors including molecular structure (which may play a role in the inhibitor accessing active sites) and the concentration of enzymes influencing the inhibitory effect of allicin.

Allicin did not affect the hemolytic activity of *P. mirabilis* hemolysin in spite of previous studies by the authors on pneumolysin O (PLY) and streptolysin O (SLO) (Arzanlou and Bohlooli 2010a; Arzanlou *et al.* 2011). It was indicated that allicin inhibits PLY and SLO by binding to the cysteinyl residue in their binding sites (Arzanlou and Bohlooli 2010a; Arzanlou *et al.* 2011). Unlike PLY and SLO, the *P. mirabilis* hemolysin active site does not contain free cysteine residues in its structure, so the ineffectiveness of allicin on *P. mirabilis* hemolysin activity could be explained by the lack of free cysteine residues in its structure (Weaver *et al.* 2009). Beside the chemical inactivation of bacterial virulence factors by allicin, other studies showed that sub-MIC concentrations of allicin could reduce the production of *Staphylococcus aureus* hemolysin (α toxin) at the genomic level (Leng *et al.* 2011).

The other experimental approach of the current study was to examine the effects of allicin on biofilm formation by *P. mirabilis* and its effect on established biofilms. Previous studies have shown that allicin could reduce biofilm formation in *S. epidermidis*, *P. aeruginosa* and *Candida albicans* at sub-MIC concentrations (Perez-Giraldo *et al.* 2003; Khodavandi *et al.* 2011; Lihua *et al.* 2013). The current study showed that allicin also can inhibit biofilm development in *P. mirabilis* at sub-MIC concentrations. In addition, allicin was found to be effective in inhibiting established biofilms at higher concentrations. These results are consistent with previous reports that state that bacteria are more resistant in biofilm than in planktonic growth (Patel 2005; Jacobsen and Shirtliff 2011). Biofilms in particular crystalline biofilms play a significant role in *P. mirabilis* UTIs (Jacobsen and Shirtliff 2011). Alkaline pH plays an essential role in the development of crystalline biofilms, so impeding the rise of urinary pH and subsequent crystallization could be a critical step in preventing biofilm formation. Urease inhibitors, such as flurofamide, have shown to reduce the development of crystalline biofilms, since they reduce pH and consequently reduce the deposition of calcium and magnesium salts on biofilm (Morris and Stickler 1998). As discussed earlier, allicin as a potent *P. mirabilis* urease inhibitor may prevent the development of crystalline biofilms in *P. mirabilis*. Therefore, further studies may be worthy of evaluation.

CONCLUSION

The results of this study revealed that allicin inhibited the growth of *P. mirabilis*. This compound also reduced the biofilm development and neutralized the urease enzyme of *P. mirabilis*. The main drawback of clinical application of allicin is its instability in biological fluids (Freeman and Kodera 1995). It has been previously shown that allicin is broken down in the body (Rosen *et al.* 2001), and this limits its clinical application. Some studies examined the formation of allicin on target cells inside the body using pro-drug enzyme systems (Arditti *et al.* 2005; Appel *et al.* 2011). This system may be applied to control *P. mirabilis* UTIs using allicin. However, to achieve this goal further studies are needed.

ACKNOWLEDGEMENTS

This work was performed in partial fulfillment of the requirements for an M.Sc. thesis in Microbiology (Mahsa Ranjbar Omid). We gratefully acknowledge Ardabil University of Medical Sciences for providing laboratory facilities for this work.

Conflict of interest. None declared.

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